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Stefan Mrochen · Dieter Klein · Sigrid Nikol
James R. Smith · Brian Salmons
Walter H. Günzburg

Inducible expression of p21$^{WAF\text{-}1/CIP\text{-}1/SDI\text{-}1}$ from a promoter conversion retroviral vector

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Abstract Constitutive, high-level expression of the potentially therapeutic WAF-1/CIP-1/SDI-1 gene is incompatible with cell growth. A promoter conversion retroviral vector carrying the WAF-1/CIP-1/SDI-1 gene under the transcriptional control of the glucocorticoid inducible promoter of mouse mammary tumor virus was used to infect human bladder carcinoma or feline kidney cells. Reduced cell growth due to a greater proportion of cells being in the G_0/G_1 phase of the cell cycle was observed when WAF-1/CIP-1/SDI-1 expression was activated by addition of glucocorticoid hormone. This system demonstrates the potential long-term therapeutic use of WAF-1/CIP-1/SDI-1 delivered by retroviral vectors for inhibiting the growth of rapidly proliferating cells. Moreover, the conditional expression of genes such as WAF-1/CIP-1/SDI-1 from such retroviral vectors may facilitate analysis of their function.

S. Mrochen
Institute of Molecular Virology
GSF-National Research Center for Environment and Health,
Oberschleißheim, and Medizinische Klinik I,
Klinikum Großhadern, Universität München,
Munich, Germany

D. Klein
Institute of Molecular Virology
GSF-National Research Center for Environment and Health,
Oberschleißheim, and Bavarian Nordic Research Institute,
Munich, Germany

S. Nikol
Medizinische Klinik I, Klinikum Großhadern,
Universität München, Munich, Germany

J.R. Smith
Huffington Center on Aging, Division of Molecular Virology,
and Departments of Cell Biology and Medicine,
Baylor College of Medicine, Houston, USA

B. Salmons
Bavarian Nordic Research Institute, Munich, Germany

W.H. Günzburg (✉)
Institute of Virology, University of Veterinary Sciences,
Josef-Baumann-Gasse 1, A-1210 Vienna, Austria and
Institute of Molecular Virology
GSF-National Research Center for Environment and Health,
Oberschleißheim, Germany

WALTER H. GÜNZBURG received his Ph.D. in London. After postdoctoral work in Switzerland and the United States, he headed a group at the GSF in Munich. He is presently Professor and Chairman of the Institute of Virology at the University of Veterinary Sciences in Vienna. His interests include the biology of retroviruses and development of targeted vectors for gene therapy of proliferative diseases.

STEFAN MROCHEN studied biology at the University of Regensburg and Ludwig Maximilian University in Munich, focusing on microbiology. He is presently finishing his Ph.D. in the GSF Research Center for Environment and Health at the Institute of Molecular Virology in Munich. His major research interests include viral methods of gene transfer and vector development.

Key words Retroviral vector · Conditional expression · Cell cycle control · Mouse mammary tumor virus long terminal repeat · WAF-1/CIP-1/SDI-1

Abbreviations *CIP* Cyclin-dependent kinase inhibitor · *DMEM* Dulbecco's modified Eagle's medium · *EB* Ethidium bromide · *FCS* Fetal calf serum · *LTR* Long terminal repeat · *MLV* Murine leukemia virus · *MMTV* Mouse mammary tumor virus · *SDI* Senescent cell-derived inhibitor

Introduction

Recently three cDNAs have been identified that cause growth arrest when transfected into young, actively dividing cells [1]. One of these sequences, senescent cell-derived inhibitor (SDI) 1, has also been independently cloned by other groups as (a) cyclin-dependent kinase inhibitor (CIP) 1 [2], (b) a gene, WAF1, that is induced by p53 [3], and (c) a gene involved in melanocyte differentiation [4]. Thus the same gene has a central role in a number of cellular processes that have in common the abrogation of cell proliferation, implicating this gene as being involved in cell cycle control. WAF-1/CIP-1/SDI-1 has been shown to be overexpressed in senescent cells, quiescent cells, and cultured primary cells undergoing crisis, suggesting a role in the maintenance of DNA synthesis inhibition [1]. Further, evidence has been presented that the WAF-1/CIP-1/SDI-1 mediated inhibition of DNA synthesis occurs via an inhibition of Cdk activity [5]. These findings, together with the demonstration that WAF-1/CIP-1/SDI-1 inhibits cell growth of young dividing cells, suggest that this gene may be useful for gene therapy. Expression of this gene could be used to slow the growth of rapidly proliferating cells in diseases such as restenosis, in which smooth muscle cells divide inappropriately.

Although high-level constitutive expression of WAF-1/CIP-1/SDI-1 is desirable for in vivo therapeutic use of this gene to prevent proliferation of rapidly dividing cells, in in vitro model systems WAF-1/CIP-1/SDI-1 expression may be so efficient as to prevent cell growth for the isolation of cell clones. In such in vitro model systems only cell clones that express low levels of WAF-1/CIP-1/SDI-1 would be able to grow, and we have observed this in previous experiments (S.M., W.H.G., and B.S., unpublished data). Expression of WAF-1/CIP-1/SDI-1 from an inducible vector would circumvent this problem, allowing the establishment of clones carrying the inducible WAF-1/CIP-1/SDI-1 construct in the *absence* of WAF-1/CIP-1/SDI-1 expression, followed by the characterization of the effects of inducible WAF-1/CIP-1/SDI-1 expression.

Here we report the introduction of a cDNA encoding WAF-1/CIP-1/SDI-1 into a novel type of retroviral vector (ProCon) that is able to undergo promoter-conversion after infection. The glucocorticoid hormone inducible promoter of mouse mammary tumor virus (MMTV; for a review see [6]) was chosen for this analysis. After infection of cells clones were isolated that show little or no expression of WAF-1/CIP-1/SDI-1 in the absence of glucocorticoid hormone. Subsequent treatment of these cells with such hormones resulted in enhanced WAF-1/CIP-1/SDI-1 expression. This expression is associated with a decreased growth rate and a tendency for these cells to accumulate in the G_1 phase of the cell cycle. These data suggest that in vivo expression of WAF-1/CIP-1/SDI-1 under the control of a strong *constitutive* promoter may prove useful for the treatment of proliferative disorders.

Materials and methods

Construction of ProCon SDI retroviral vector

The WAF-1/CIP-1/SDI-1 cDNA insert was isolated from the plasmid pSDI1 [1] by PCR using two primers:

- SDIn1: 5′-TATGGACGTCTCCCTGCCGAAGTCAGTT-3′
- SDIn2: 5′-TATGGGATCCGGCAGAAGATGTAGAGCG-3′

carrying *Bam*HI (bold sequence) and *Aat*II (bold sequence) restriction sites as extensions. The plasmid p125.gal, carrying a retroviral vector derived from pBAG [7], in which the whole U3 region of the 3′ long terminal repeat (LTR) was replaced with the U3 region of MMTV, was digested with *Aat*II and *Bam*HI (Fig. 1). The resulting 5-kb vector containing fragment was ligated to the SDI fragment, creating the plasmid p125.SDI (Fig. 1). In order to extend the retroviral packaging signal p125.SDI was digested with *Aat*II and *Afl*II, and the resulting 4.9-kb fragment was blunt-ended and ligated to a 3.5-kb blunt-ended *Eco*RI/*Afl*III fragment of pLXSN [8], yielding the plasmid pLXS-SDI1 (Fig. 1).

Cell culture and growth assay

EJ [9], CRFK [10], and PA317 retrovirus packaging cells [11] were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). For growth studies 5000 EJ or CRFK cells were plated in each well of a six-well plate (Falcon) in DMEM containing 10% FCS either with or without 10^{-6} M dexamethasone. After 7 days the cells were fixed in methanol:acetic acid (3:1) and stained with Giemsa (Fisher So-G-28).

Transfections and infections

The cells were seeded in 10-cm tissue culture dishes at a density of 2×10^6 cells 1 day prior to lipofection and then lipofected with 4 µg plasmid and 2 µg pHCMV-G [12] using the lipofectamine kit from GIBCO/BRL according to the manufacturers instructions. Infections were performed essentially as described previously [12, 13]. Briefly, 24-h conditioned cell culture medium from 2×10^6 125SDI/PA317 cells was passed through 0.45 µm Millipore filters, polybrene added to a final concentration of 8 µg/ml and 2 ml used to infect 1×10^6 EJ cells in a 10-cm culture. After 4 h incubation 6 ml medium was added and the cells cultured overnight. The cells were trypsinized and one-fifth of the total amount of cells was seeded in a new culture dish. After 24 h the cell culture medium was changed to DMEM containing 800 µg/ml G418, and 14 days later resistant cell clones were isolated.

PCR analysis and hybridization

Genomic DNA was prepared [14] and used for PCR. Each PCR reaction contained 1 µg genomic DNA and 0.4 µM of each primer. MMTV U3 specific primers (A: 5′-TAGGTGGGTCACAATCAAC-3′; B: 5′-GACCACAGCCAACTTCCTCTTACA-3′) or a murine leukemia virus (MLV) R region specific primer (C: 5′-GCGCCAGTCTTCCGATA-3′) or an MLV U3 primer (E: 5′-CAGTTCGCTTCTCGCTTCTGTTCG-3′) was used in combination with an SDI coding sequence specific primer (D: 5′-CGGCTCAACAAGGAACTGAC-3′). PCR reactions were performed in a Perkin Elmer Cetus thermal cycler 9600 for 35 cycles (each cycle: 1 min at 94°C for denaturation; annealing at 55°, 53°, 56°, or 57°C for primer pairs A+D, B+D, C+D, and E+D, respectively; elongation at 72°C for 5 min). PCR products were separated on 0.8% agarose gels and then transferred onto nylon filters (Zeta probe GT, Bio-Rad, Munich). Hybridization probes were ^{32}P-labeled using a random primer kit (Pharmacia, Ready-to-go). Prehybridization and hybridization of the probe to the filter bound PCR products and filter washing was performed according to the milk powder meth-

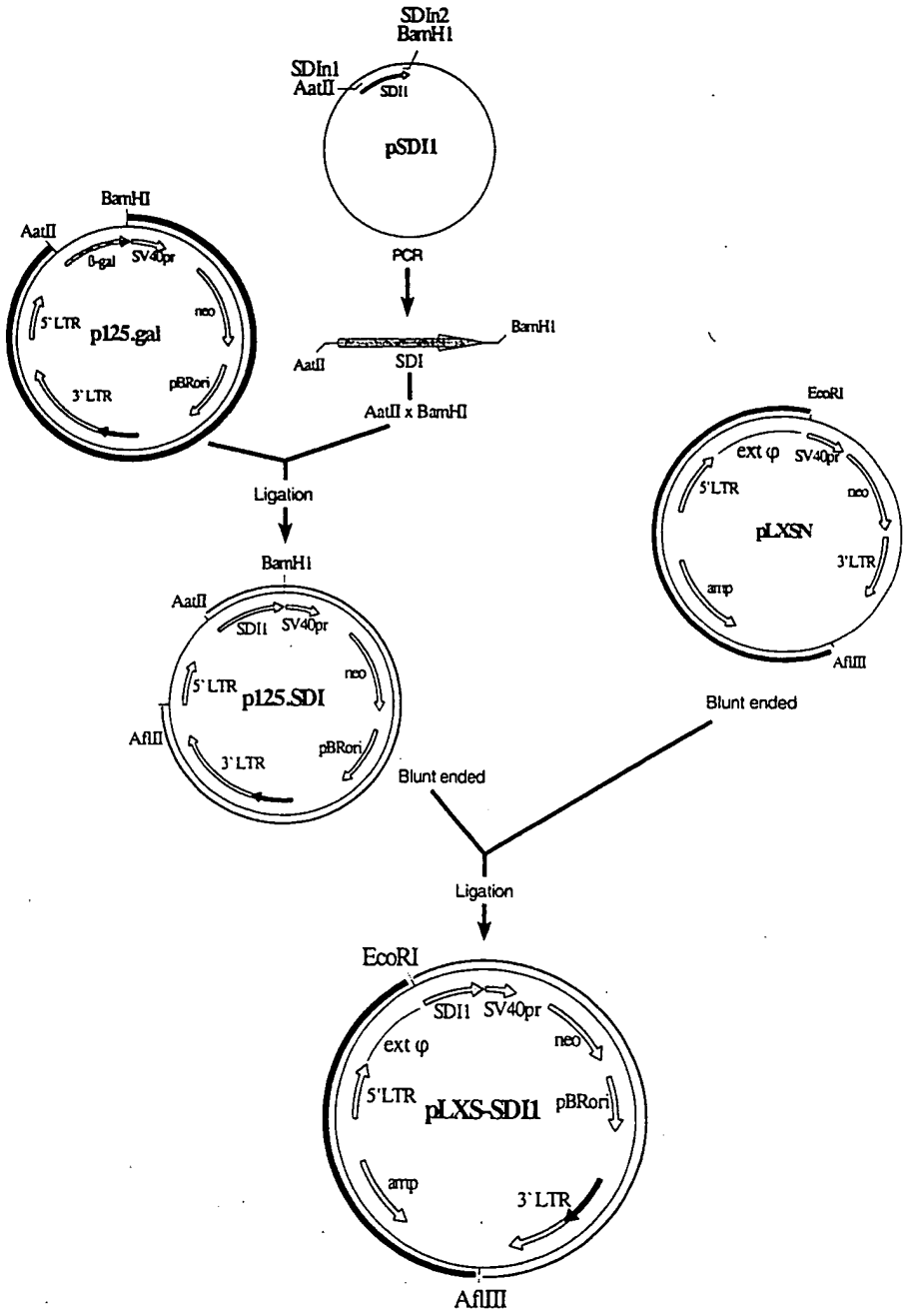


Fig. 1 Retroviral vector cloning strategy. A 666-bp fragment containing the *SDI1* gene and *Aat*II and *Bam*H1 restriction sites as indicated, was amplified by PCR using pSDI1 as a template and SDIn1 with SDIn2 as primers. The fragment was cloned into p125.gal, using *Aat*II and *Bam*H1 restriction sites, thereby replacing the β-galactosidase gene with WAF-1/CIP-1/SDI-1. The new vector was named p125.SDI. After digesting p125.SDI with *Aat*II and *Afl*II, the 4911-bp fragment was isolated, blunt-ended, and ligated with a blunt-ended 3529-bp fragment resulting from a *Eco*RI/*Afl*III digest of pLXSN. The new vector was named pLXS-SDI1. Restriction sites shown are ones used for cloning. *Black arrows*, MMTV-U3 region containing the inducible promoter; *Ext*, the location of the extended packaging signal; *neo*, *amp*, location of the neomycin and ampicillin resistance genes, respectively; *pBRori*, location of the origin of replication; *SV40pr*, location of the SV40 promoter

od [14]. Hybridization signals were visualized using a Fuji phosphoimaging system (BAS 1000).

Fluorescent activated cell scanning

EJ cells (5×10^5) were seeded into T-225 flasks and cultured in DMEM containing 3% FCS with or without 10^{-6} M dexamethasone (Sigma, Germany). To avoid growth inhibition due to cell-to-cell contact cells were grown only until they reached 40–50% confluence. For cell cycle analysis the DNA was stained according to the micronuclei method [15]. In brief, cells were trypsinized (1× trypsin, Gibco/BRL, GB) and washed once with phosphate-buffered saline. Cells (10^6) were resuspended in 1 ml solution 1 [10 mM NaCl, 3.4 mM Na-citrate, 10 mg/l RNase A (Serva, Germany); 0.3% Nonidet P-40 and 25 mg/l ethidium bromide (EB; Sigma, Germany)]. After 60 min of incubation at room temperature 1 ml solution 2 (0.07 mM citric acid, 0.25 M sucrose, and 40 mg/l EB) was added and the cells kept on ice until measurement. Fluorescent-activated cell scanning was performed with an FACSCalibur [Becton Dickinson; excitation of EB with an argon laser, 488 nm; EB fluorescence was detected with a long pass filter (LP 640)]. A first gate was set to exclude unspecific debris from the nuclei according to side scatter and forward scatter. A gate in the fluorescence 2 width–fluorescence 2 area dot plot was used to exclude doublets. The DNA content was estimated using a fluorescence 2 area histogram. Cell cycle distribution was analyzed using the MOD FIT LT program (Verity Software House).

S1 nuclease protection

Total RNA was isolated from cells grown in the presence or absence of 10^{-6} M dexamethasone and 40 µg hybridized to a ^{32}P end

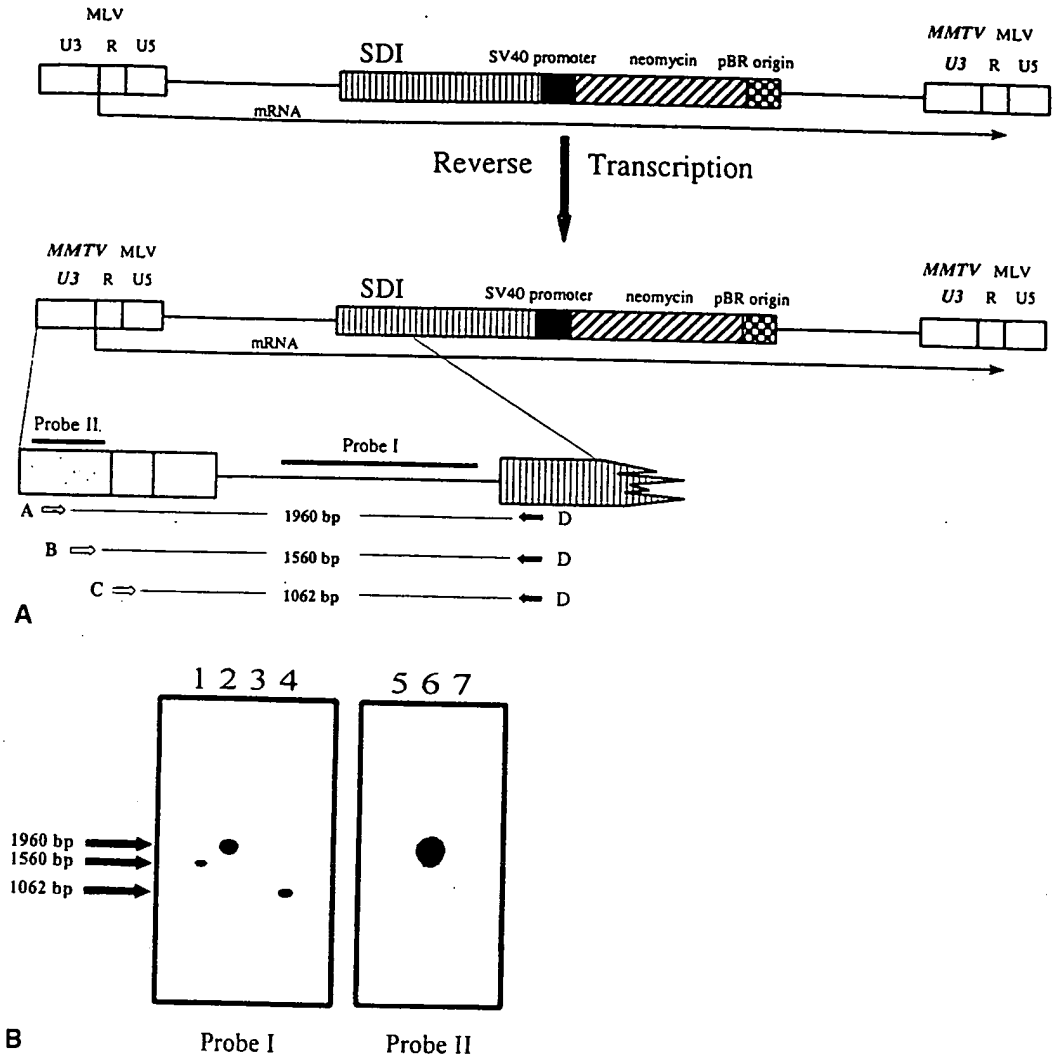


Fig. 2A, B DNA analysis of transduced cells. **A** Schematic representation of the retroviral vector pLXS-SDI1 before and after infection followed by reverse transcription. MLV (*open boxes*) and MMTV (*dotted boxes*) derived U3 regions as well as MLV derived R and U5 regions, the SDI gene, pBR origin, SV40 promoter, and neomycin resistance gene are shown. The U3, R, and U5 region comprise the LTR. The enlarged region of the retroviral vector shows the approximate locations of the primers used for PCR amplification, the sizes of their products and the radioactive probes I (*Aat*II×*Eco*RI) and II (*Pst*I) used for hybridization. **B** Hybridization of PCR products which are derived from human genomic DNA. The DNA was isolated from a human bladder carcinoma (EJ) cell line which had been infected with the retroviral vector pLXS-SDI1 (see Fig. 1). Primer pairs used for PCR: B/D, *lane 1/5*; A/D, *lane 2/6*; D and MLV U3 specific primer E (location not shown), *lane 3/7*; C/D, *lane 4*. PCR products were hybridized with probe I (*lanes 1–4*) or II (*lanes 5–7*). The expected DNA fragments of 1560 bp and 1960 bp were detected using probe I (*lanes 1, 2*) or probe II (*lanes 5, 6*) only after successful promoter conversion. The DNA fragment of 1062 bp is detectable as expected even without promoter conversion but only with probe I (*lane 4*). *Lanes 3 and 7*, no fragment is detectable either with probe I or with probe II because the complementary sequence for the MLV U3 specific primer E is not present after successful promoter conversion

labeled 1.6 kb *Bsa*I MLV-specific DNA fragment (Fig. 3), as previously described [13]. Briefly, after hybridization overnight at 45°C, the samples were digested with S1 nuclease (80 U, 1 h), phenol extracted to remove protein and precipitated in 70% ethanol. The pellets were dried, resuspended in Sanger sequencing buffer, and separated on a 6% denaturing polyacrylamide gel.

Western blotting

Total cell protein extracts were isolated and separated on 14% SDS-PAGE gels as described previously [13]. After transfer of the proteins to nitrocellulose (Schleicher & Schüll, BA83 0.2 µm) using the semidry transfer method with a BioRad Trans Blot SC, nonspecific binding sites were blocked with 1% BSA in TBST (10 mM Tris pH8.0, 150 mM NaCl, 0.05% Tween 20). The nitrocellulose filter was incubated with a 1:500 dilution (TBST) of a monoclonal mouse antibody that recognizes a determinant of $p21^{WAF-1/CIP-1/SDI-1}$ (Santa Cruz Biotechnology, Santa Cruz, USA) for 90 min at room temperature. The filter was then washed three times with TBST and incubated with anti-mouse antibodies coupled to alkaline phosphatase for 60 mins. After three TBST washes the filter was transferred into a developing solution consisting of 198 µl nitroblue tetrazolium (50 mg/ml in 70% dimethyl formamide), 99 µl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 70% dimethylformamide) in 30 ml AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), and the color development reaction was stopped with distilled water.

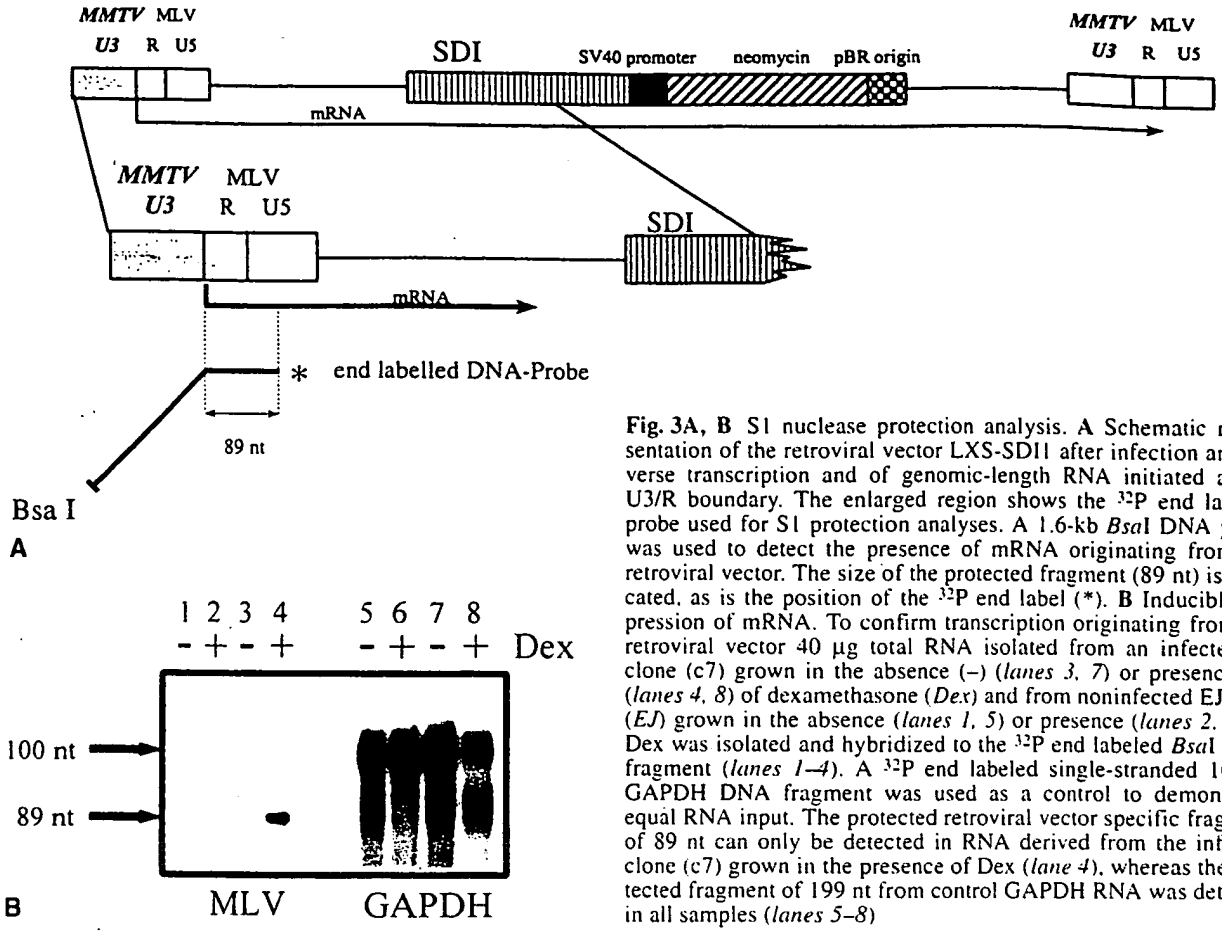


Fig. 3A, B S1 nuclease protection analysis. **A** Schematic representation of the retroviral vector LXS-SDI1 after infection and reverse transcription and of genomic-length RNA initiated at the U3/R boundary. The enlarged region shows the ^{32}P end labeled probe used for S1 protection analyses. A 1.6-kb *Bsa*I DNA probe was used to detect the presence of mRNA originating from the retroviral vector. The size of the protected fragment (89 nt) is indicated, as is the position of the ^{32}P end label (*). **B** Inducible expression of mRNA. To confirm transcription originating from the retroviral vector 40 μg total RNA isolated from an infected EJ clone (c7) grown in the absence (–) (*lanes 3, 7*) or presence (+) (*lanes 4, 8*) of dexamethasone (*Dex*) and from noninfected EJ cells (*EJ*) grown in the absence (*lanes 1, 5*) or presence (*lanes 2, 6*) of Dex was isolated and hybridized to the ^{32}P end labeled *Bsa*I DNA fragment (*lanes 1–4*). A ^{32}P end labeled single-stranded 100 nt GAPDH DNA fragment was used as a control to demonstrate equal RNA input. The protected retroviral vector specific fragment of 89 nt can only be detected in RNA derived from the infected clone (c7) grown in the presence of Dex (*lane 4*), whereas the protected fragment of 199 nt from control GAPDH RNA was detected in all samples (*lanes 5–8*)

Results

A promoter-conversion (ProCon) retroviral vector carrying an inducible SDI gene

Elevated expression of WAF-1/CIP-1/SDI-1 is associated with G_1 arrest and cell senescence. However, our original observations suggested that *constitutive* expression of WAF-1/CIP-1/SDI-1 selected for a population of cells that had downregulated WAF-1/CIP-1/SDI-1 expression (S.M., B.S., and W.H.G., unpublished data). Accordingly, we utilized a novel class of retroviral vector (ProCon) in which the inducible MMTV promoter is inserted into the vector construct in place of the normal retroviral promoter carried in the 3′ LTR [16]. This allows expression of the retroviral genomic RNA molecule from the MLV promoter in the 5′ LTR in retroviral packaging cells, but as a consequence of reverse transcription the conditional MMTV promoter is used to drive the expression of the SDI cDNA in infected cells (pLXS-SDI1; Fig. 1).

Human bladder derived EJ cells and feline kidney (CRFK) cells were infected with the LXS-SDI1 retroviral vector and G418-resistant cell clones obtained. Genomic DNA was prepared from a number of clones and PCR performed to demonstrate that they had acquired the retroviral construct, and that promoter conversion

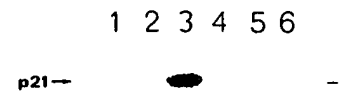
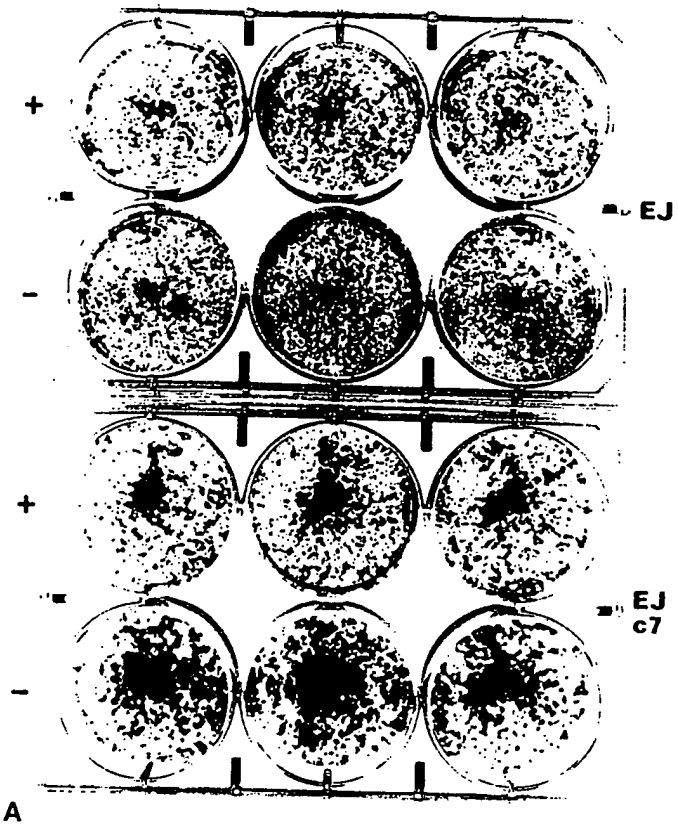


Fig. 4 Inducible expression of the p21 SDI1 protein. To confirm the presence of the p21 SDI-1 protein coded by the LXS-SDI-1 vector total protein from two CRFK clones (c2, *lanes 1, 2*; c5, *lanes 3, 4*) grown in the presence (*lanes 1, 3*) or absence (*lanes 2, 4*) of Dex and total protein from noninfected CRFK cells grown in the presence (*lane 5*) or absence (*lane 6*) of Dex was extracted and used in a western blot using mouse anti-p21 SDI-1 monoclonal antibody. p21 SDI-1 protein can be detected only in infected CRFK clones (c2 and c5) grown in the presence of Dex (*lanes 1 and 3*, respectively)

had occurred. PCR products of 1960 and 1560 bp were obtained using either of two MMTV-specific primers (Fig. 2A; primers A and B) in combination with a second primer (Fig. 2A; primer D) specific for WAF-1/CIP-1/SDI-1 coding sequences. These products hybridized to MLV-Ψ region [(Fig. 2B, probe I, lanes 1 (primers B and D) and 2 (primers A and D)] and MMTV-U3 [probe II, lanes 5 (primers B and D) and 6 (primers A and D)] specific probes. The product in lane 5 hybridizes only very weakly to the MMTV-U3 probe since only one-fifth of probe II is homologous to the PCR product. A 1062-bp



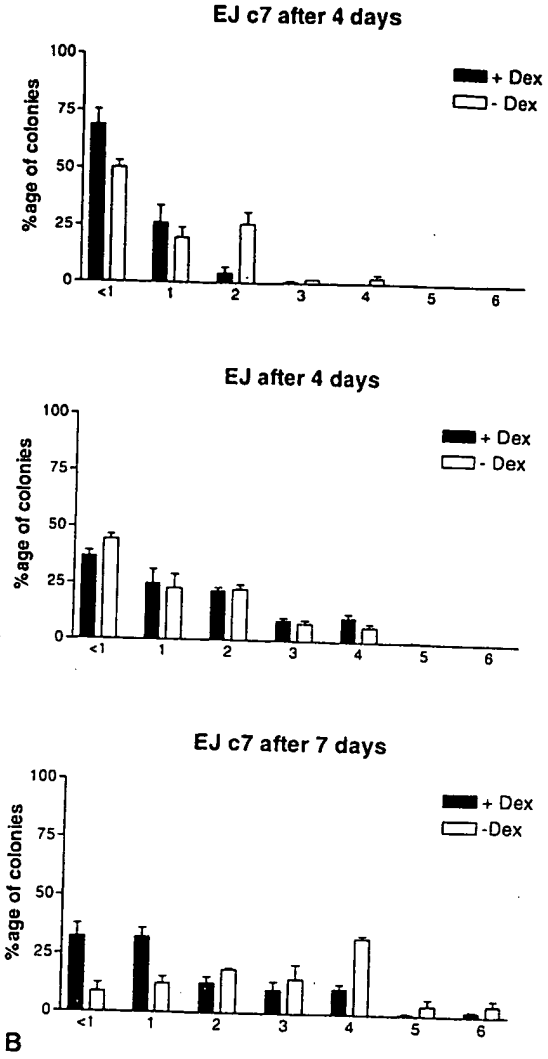


Fig. 5A, B Growth regulatory potential of p21 SDI1 protein. To determine the effect of p21 SDI1 protein expression on growth of EJ cells, 5000 EJ or EJ infected with pLXS-SDI1 (clone 7) cells were seeded in each well of a six-well plate, and Dex was added (+) or not (–) as indicated. After 7 days the cells were fixed and stained with Giemsa. **A** Less cell growth was detectable when the pLXS-SDI1 infected clone 7 was grown in the presence of Dex than in the absence of the synthetic hormone. **B** A quantitative analysis of the growth effects of SDI expression was determined by counting the number and size (area) of colonies after growth in the absence and presence of dexamethasone. Shown are the percentages of colonies of the given size classes. Each size class unit (*x-axis*) represents an area of 2 mm×2 mm at a magnification of ×32

PCR product generated using a primer specific for the MLV-R region (Fig. 2A, primer C) in combination with the SDI primer (Fig. 2A, primer D) hybridized only to the MLV probe (Fig. 2, lane 4) and not with the MMTV probe (lane 7), as expected. No products were observed using MLV-U3 specific primers in conjunction with the WAF-1/CIP-1/SDI-1 primer (primer D; Fig. 2, lane 3). These data verify that promoter conversion had indeed occurred, placing the SDI coding sequences under the control of the dexamethasone-inducible MMTV promoter.

Inducible expression of WAF-1/CIP-1/SDI-1 in infected cells

To show that transcription of mRNA is under control of the MMTV promoter and thus can be enhanced by the addition of dexamethasone RNA was prepared from the clones and used for an S1 nuclease analysis. The ^{32}P end labeled probe used should protect an 89 nt fragment which corresponds to sequences from the R and part of the U5 region of the MLV LTR (Fig. 3, panel A). This fragment was protected by RNA from a representative EJ clone (clone 7) only when these cells were grown in the presence of dexamethasone (Fig. 3, panel B, lane 4). No protected fragment was seen using RNA from the same cells grown in the absence of dexamethasone (lane 3), indicating that RNA expression was indeed induced by the synthetic glucocorticoid. Other clones showed basal expression in the absence of dexamethasone which was enhanced by hormone treatment (not shown), as previously observed when using the MMTV LTR for conditional expression of heterologous genes [17, 18].

The inducibility of WAF-1/CIP-1/SDI-1 expression was also observed at the protein level. Proteins were ex-

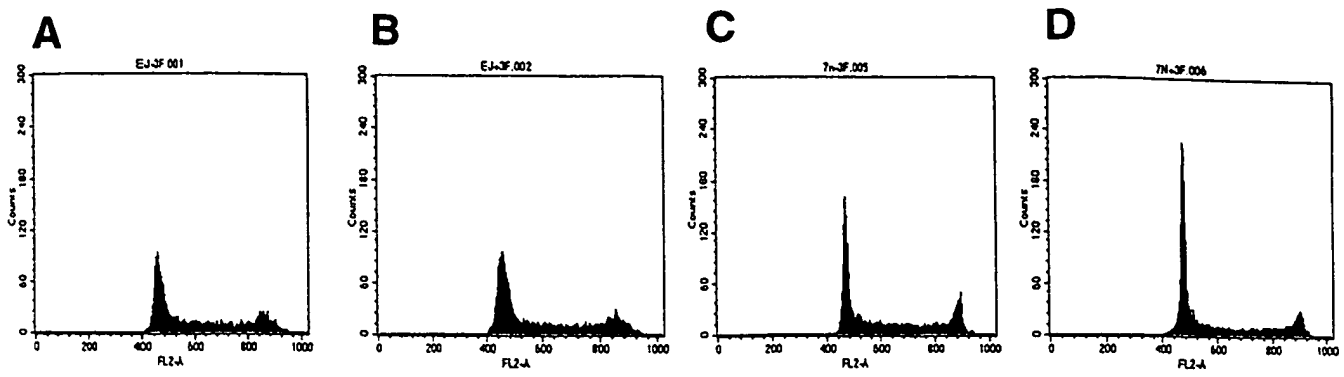


Fig. 6 Fluorescent-activated cell scanning of proportion of cells in the stages of the cell cycle. EJ cells (**A**, **B**) and an EJ SDI clone (**C**, **D**) were grown either in the absence (**A**, **C**) or presence (**B**, **D**) of 10^{-6} M dexamethasone. The DNA content of cells was estimated using the fluorescence 2 area histogram, and the cell cycle distribution is shown

tracted from two of the infected CRFK cell clones (clones 2 and 5) grown either in the presence (Fig. 4, lanes 1 and 3) or absence (lanes 2 and 4) of dexamethasone and analyzed by western blotting using a monoclonal antibody specific for the p21$^{WAF-1/CIP-1/SDI-1}$ protein. This protein was detected only after treatment of the cells with dexamethasone (Fig. 4, lanes 1 and 3).

Growth properties of infected cells

In order to test whether the induction of p21$^{WAF-1/CIP-1/SDI-1}$ expression is associated with reduced cell proliferation, infected EJ cells were sparsely seeded in medium containing or lacking dexamethasone, and after 7 days the cells were fixed and stained. Infected EJ cell clone 7 (Fig. 5A, c7) showed less growth after induction of WAF-1/CIP-1/SDI-1 expression by dexamethasone treatment (Fig. 5A, c7+) than its nontreated counterpart (Fig. 5A, c7–). This effect is not due to the dexamethasone since control noninfected cells do not show reduced growth in the presence of dexamethasone (Fig. 5A, EJ+). The growth inhibitory effects of SDI expression were quantitatively assessed (Fig. 5B). The percentage of colonies of different sizes was compared for EJ cells and infected EJ cell clone 7 cells grown in the presence (filled boxes) or absence (open boxes) of dexamethasone after 4 and after 7 days growth. After 4 days in culture in the presence of dexamethasone the colonies of EJ cells infected with the SDI virus (EJc7) were smaller (approx. 70% of the cells in size group <1) than the noninfected (EJ) cells (approx. 37% of the cells in size group <1). The addition of dexamethasone only marginally affected the size of colonies of the noninfected cells (compare filled and open bars in the EJ graph). In contrast, a 20% increase in the number of smaller colonies was visible for the SDI-infected cells, after 4 days growth (compare filled and open bars in the EJc7 graph). After 7 days the noninfected EJ cells reached confluency. Colonies from the SDI infected cells (EJc7), however, could still be distinguished, and these cells grown in the presence of dexamethasone showed a dramatic shift towards the smaller size range (filled bars) than the same cells grown in the absence of dexamethasone (open bars).

Fluorescence-activated cell scanning of infected cell clones was also performed to determine the proportion of cells in various stages of the cell cycle. Dexamethasone treatment of EJ cells results on average in a nonspecific 4.7% increase in the number of cells in the G_0/G_1 phase and a 4.2% decrease in the number in the S phase. Figure 6 presents a typical experiment showing a nonspecific increase of 2.4% in the G_0/G_1 phase (compare panels A and B). Dexamethasone treatment of the representative infected EJ clone 7 in this experiment resulted in a 13.3% increase of cells in the G_0/G_1 phase and a decrease of 10.3% for cells in the S phase (Fig. 6; compare panels C and D). The average increase of cells in the G_0/G_1 phase that we have recorded is 11.9%. A similar effect was seen with the CK cell clones. Dexamethasone treatment of CK cells results in faster cycling and thus a 11% decrease in the percentage of cells in the G_0/G_1 phase. In contrast, two clones of infected CK cells expressing p21$^{WAF-1/CIP-1/SDI-1}$ after dexamethasone treatment showed an average 5% increase of the percentage of cells in the G_0/G_1 phase (not shown). Taking into account the stimulatory effect of dexamethasone on these cells, this converts to an increase in the G_0/G_1 phase of about 15%.

Discussion

p21$^{WAF-1/CIP-1/SDI-1}$ is associated with checkpoint control during progression through the cell cycle. Progressive elevation of WAF-1/CIP-1/SDI-1 expression is also seen as cells lose their proliferative capacity during senescence in culture [1]. These findings suggest that the constitutive overexpression of p21$^{WAF-1/CIP-1/SDI-1}$ is useful for gene therapy approaches to treat diseases that are charac-

terized by rapid proliferation of cells such as is found in certain cancers and vascular diseases such as restenosis [19].

Retroviral vectors are ideal gene transfer vehicles for stable therapeutic gene delivery to rapidly dividing cells. These vectors are usually derived from MLV, and the therapeutic gene is expressed either from the MLV promoter or from a second internal heterologous promoter [20]. In initial experiments we inserted the WAF-1/CIP-1/SDI-1 cDNA into a retroviral vector under the control of the constitutively active MLV promoter. Introduction of this construct into cells in culture, by either transfection or infection, resulted in fewer than expected colonies and low or undetectable expression of WAF-1/CIP-1/SDI-1 in isolated clones, probably since high-level expression of this gene product is not compatable with cell growth. Nevertheless, some of these clones showed a reduced ability to form tumors when injected into nude mice (S.M., B.S., and W.H.G., unpublished data).

The glucocorticoid inducible promoter located within the MMTV LTR has been coupled to the coding sequences of many different genes, and the effects of gene expression can therefore be analyzed in the same cell before and after glucocorticoid induction of gene expression [6]. We have recently constructed a novel ProCon retroviral vector, based upon MLV, in which the MLV retroviral promoter used in the infected cell can be replaced by any promoter of interest [16]. In this study such a vector was modified to carry WAF-1/CIP-1/SDI-1 coding sequences under the transcriptional control of the MMTV promoter after infection.

A number of cell clones infected with this retroviral vector were obtained and analyzed. It was demonstrated that promoter conversion actually occurs in the infected cells, and the WAF-1/CIP-1/SDI-1 gene was thus placed under the transcriptional control of the MMTV promoter (Fig. 2). Further, the expression of WAF-1/CIP-1/SDI-1 was upregulated by treatment of the cells with the synthetic glucocorticoid dexamethasone (Figs. 3, 4). Induction of $p21^{WAF-1/CIP-1/SDI-1}$ expression was associated with reduced proliferation (Fig. 5), an increased number of cells in the G_0/G_1 phase of the cell cycle, and a corresponding decrease in the number of cells in the S phase. Taken together these data verify that overexpression of $p21^{WAF-1/CIP-1/SDI-1}$ inhibits cell proliferation. In the absence of dexamethasone the MMTV promoter is weakly active; however, addition of dexamethasone augments promoter activity and can give expression levels typical of a constitutive promoter, such as MLV, in some clones [17, 18]. Thus the levels of expression obtained in this study, although high enough to show moderate growth inhibitory effects, could theoretically be increased even further if stronger promoters were used. For actual gene therapy this would be both desirable and advisable.

Although retroviral vectors based upon MMTV have been constructed for gene transfer, current generations of these vector systems are inefficient, in part due to the more complex regulation of MMTV than that of MLV. Such vectors may also deliver genes to only certain cell types [6]. Nevertheless MMTV vectors have been shown to give inducible expression of therapeutic genes, such as tumor necrosis factor-α, albeit after transfection [18]. The alternative approach taken here combines the relatively efficient MLV vector system with the inducible MMTV promoter. The replacement of the MLV promoter with that of MMTV rather than the internal insertion of the MMTV promoter in addition to the MLV promoter may avoid the problem of promoter interference that often results in the loss of expression from the MLV promoter (discussed in [20]).

Overhauser and Fan inserted a 335-bp fragment from the MMTV LTR carrying the glucocorticoid response elements into the MLV LTR and generated infectious recombinant MLV used for infection of NIH3T3 cells. Dexamethasone treatment of cells infected with this hybrid virus resulted in only a two- to fourfold increase in expression [21]. This is presumably due to interference between the MLV enhancers and the MMTV sequences. In our studies reported here the MLV promoter was completely replaced with that of MMTV, and an induction of expression at least ten-fold was observed. This is similar to the expression induction that was previously seen from recombinant constructs in which the complete MMTV LTR is linked to heterologous coding sequences [6, 17, 18].

Recently it has been shown that $p21^{WAF-1/CIP-1/SDI-1}$ is involved in normal proliferation control of smooth muscle cells [22], and adenoviral vector mediated delivery of this gene to these cells or to tumor cells has antiproliferative effects [22, 23]. However, these vectors, in contrast to retroviral vectors, do not integrate their DNA into the cell DNA and eventually may be lost [24]. Regardless of the vector system used, long-term therapeutic benefit may be possible only if all or the vast majority of tumor cells receive and express WAF-1/CIP-1/SDI-1 since there is no evidence of a bystander effect [23]. Our studies presented here, using a conditional retroviral vector expressing $p21^{WAF-1/CIP-1/SDI-1}$ in an inducible manner, have allowed direct demonstration that in the same cell expression of WAF-1/CIP-1/SDI-1 results in growth inhibition as evidenced by reduction in colony size and an increased proportion of cells in the G_0/G_1 stage of the cell cycle. Clearly such an inducible vector provides a useful tool to examine the mechanism by which SDI inhibits cell proliferation and influences the differentiation and aging status of cells.

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